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Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: MONOCLONAL ANTIBODIES AND THEIR USE.

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Agent's address Coomb House
7, St. John's Road
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Agent's ADP number 03981701001 1800001

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5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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8 Checklist

8 Please supply duplicates of claim(s), abstract, description and drawing(s).

8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77	no		
Claim(s)	1	Description	15
Abstract	1	Drawing(s)	7

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Monoclonal antibodies and their use

1. Background

Acquired immune deficiency syndrome (AIDS) is recognized as a distinct disease whose etiology has been identified as being associated with infection of a lymphotrophic retrovirus termed human immunodeficiency virus (HIV). The disease is characterized by a disorder associated with an impaired cell-mediated immunity and absolute lymphopenia, particularly reduced helper T lymphocytes (CD4). AIDS may be preceded by a presyndrome that is usually manifested by a complex of designated clinical features and helper T lymphopenia. The presyndrome is called AIDS-related complex (ARC).

HIV belongs to a group of viruses that have been intensively studied over the past two decades. When a retrovirus invades a human or animal cell, the RNA is turned into DNA and inserted into the host cell, which is then duped into treating the virus's genes as its own. HIV can remain latent in these cells for years, safe from attack by the body's immune system and blindly copied each time the host cell divides. Only in case of triggering rapid viral replication by activation of the infected cells the produced virus particles kill these cells and spill into the bloodstream.

Because of the specific features of HIV a cure by elimination of both the virus and the proviral genetic information already transcribed into the human genom from already infected patients is hard to achieve. Therefore, most therapeutic attempts have been concentrated on agents that slow down the development of the disease by interfering with essential steps for the viral replication.

Prevention of HIV-infection and therapeutic intervention in already infected patients by means of a safe and effective vaccine is a major goal. Several vaccine approaches are tested in preclinical or early clinical phases. They are mainly based on viral structures as antigen, particularly on the major envelope glycoprotein gp120.

The naturally occurring immune response to the virus consists of antibodies to all viral proteins as well as activation of the cellular immunity. However, this host reaction to the HIV-infection does not appear to finally halt the progress of the disease after an asymptomatic phase which frequently lasts for years. Therefore, vaccination approaches based on the same antigens which cause the naturally occurring and finally non-protective immune response remain doubtful. One problem is the extensive heterogeneity of HIV by which this virus escapes from the attack of type-specific neutralizing anti-gp120 antibodies.

Effective protection of HIV by vaccination requires two defense strategies: one against free virus travelling in the bloodstream, and another against cells that are already infected. It is

known that HIV-infected cells in vitro and in vivo express on their surface an altered glycosylation pattern, namely the Lewis Y carbohydrate determinant. As this antigen normally occurs only during certain fetal development stages and is also associated with a variety of malignancies, the expression on HIV-infected cells may reflect their altered differentiation status induced by retroviral transformation. Therefore, this surface phenotype resembles an unique cellular host reaction to the transfection of the human genom by HIV.

The HIV envelope glycoprotein is performed by the glycosylation machinery of the infected cells. Therefore, changes in the glycosylation pattern of infected cells producing HIV are also found on free released virus particles. In consequence, the envelope glycoprotein of HIV generated in such cells also consists of Lewis Y carbohydrate determinants. Thus, the Lewis Y oligosaccharide represents a specific host response expressed both on HIV-infected cells and free HIV-particles.

Based on the above described considerations the Lewis Y structure fulfils all requirements for the use in a vaccination strategy against both free virus and HIV-infected cells. Furthermore, being a host reaction to HIV in general, this antigen is independent of HIV strain and not influenced by the genetic variability of this virus. Unfortunately, based on its carbohydrate structure and its "self" properties as fetal differentiation antigen, Lewis Y is almost not immunogenic by itself. No natural immune response against this antigen is detected in man.

One approach towards manipulating the immune system is based on idiotypic interactions. The unique antigenic determinants in and around the antigen-combining site of an Ig molecule which make one antibody distinct from another are defined as idiotopes. The totality of all idiotopes present on the variable portion of a given antibody is referred to as its idiotype (id). The molecular structure of an idiotype has been localized to both the complementarity determining regions and the framework regions of the variable domain and is generally but not always contributed to by both the heavy and the light chains in specific association.

Idiotypes are serologically defined entities since injection of an antibody (often referred to as Ab1) into a syngeneic, allogeneic, or xenogeneic recipient induces the production of anti-idiotypic antibodies (often referred to as Ab2). Based on the assumption that idiotype/anti-idiotype interactions exist physiologically a receptor-based regulation of the immune system was postulated by Niels Jerne (Ann. Immunol. 125C, 373, 1974). His network theory views the immune system as a collection of Ig molecules and receptors on T lymphocytes, each capable of recognizing an antigenic determinant (epitope) through its combining site (paratope), and each capable of being recognized by other antibodies or cell-surface receptors of the system through the idiotopes that it displays. Many studies have indeed demonstrated that idiotypic

and anti-idiotypic receptors are present on the surface of both B- and T-lymphocytes as well as on secreted antibodies.

When the binding between Ab1 and Ab2 is inhibited by the antigen to which Ab1 is directed, the idiotype is considered to be binding-site-related, since it involves a site on the antibody variable domain that is engaged in antigen recognition. Those idiotopes which conformationally mimic an antigenic epitope are called the internal image of that epitope. Since both an Ab2 and an antigen bind to the relevant Ab1, they may share a similar three-dimensional conformation which represents the internal image of the given antigen.

Carbohydrate antigens in general evoke a very pure immune response. In contrast, internal image anti-idiotypic antibodies expressing three-dimensional shapes which resemble structural epitopes of the respective carbohydrate determinant are recognized as foreign molecule. Therefore, the immune response raised by vaccination with internal image anti-id antibodies may cross-react with the original carbohydrate antigen.

Monoclonal antibodies with specificity of BR55-2 (disclosed in e.g. Wistar EP 285 059, M.Blaszcyk-Thurin et al., J. Biol. Chem. 262 (1987) 372-379, or Z. Steplewski et al., Hybridoma 9 (1990) 201-210) define the Lewis Y antigen. This carbohydrate determinant is selectively expressed on a majority of solid tumors. As mentioned above, it also occurs on HIV infected cells and on free HIV produced by these cells.

The Lewis Y oligosaccharide determinant which is also expressed during certain stages of embryonic development is almost not immunogenic by itself. However, monoclonal anti-idiotypic antibodies (Ab 2) against BR55-2 (Ab1) with internal image properties by resembling structural epitopes of the Lewis Y antigen may be useful for induction of immunity against both free HIV and HIV-infected cells independent of virus strain.

The present invention comprises the generation, production and characterization of murine monoclonal internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1) and the use of these anti-idiotypic Mabs for therapeutic and prophylactic vaccination against diseases caused by HIV.

2. Generation and characterization of murine monoclonal anti-idiotypic antibodies against the idiotype of antibodies BR55-2

In an attempt to minimize undesired anti-isotypic immune responses, the $F(ab')_2$-fragment of BR55-2, murine IgG3, was chosen for immunization. For the successful generation of murine anti-id Mabs against the idiotype of the murine Mab BR55-2, it is important to maximize the immunogenicity in order to raise an appropriate immune response in the syngeneic host. Therefore the $F(ab')_2$-fragment which is devoid of the Fc-part (cleavage and purification de-

scribed in W 092/03165) was coupled to Keyhole Limpet Hemocyanin (KLH) as immunogenic carrier using the heterobifunctional linker N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia) according to described methods (J. Carlsson et al., Biochem. J. 173, 723, 1978).

Balb/c mice were immunized with this BR55-2/murine IgG3-F(ab')$_2$-KLH-conjugate using Freund's complete adjuvant based on a typical protocol for the generation of murine Mabs. Following repeated immunizations the murine spleen cells were fused with the murine myeloma cell line SP2/0 (for experimental details see example 1).

For an appropriate selection of the cultured hybridoma cells a sequence of tests of their supernatants was performed. This selection was based on the following criteria:

a) Secretion rate of hybridomas by determination of the concentration of murine IgG in the supernatants (for experimental details see example 2). Cells producing high amounts of murine IgG were subcloned to single cell cultures.

b) Binding of selected supernatants to the F(ab')$_2$-fragment of BR55-2/murine IgG3 (for experimental details see example 3).

c) Inhibition of binding of BR55-2/murine IgG2a to Lewis Y antigen positive SKBR5 human breast cancer cells by selected supernatants (for experimental details see example 4).

The latter test is designed to be indicative for internal image properties of Ab2's. The murine IgG2a switch variant of BR55-2 was used for binding in order to minimize detection of Ab2's recognizing remaining constant regions of the F(ab')$_2$-fragment of BR55-2/murine IgG3 used for immunization. This test was performed in a quantitative manner based on the IgG concentration determined in test a) (example 2). Furthermore an excess of unspecific mouse IgG was added to this inhibition experiment in order to avoid any detection of Ab2's not specific for the idiotype of BR55-2.

Hybridomas were chosen which produce IgG with an inhibition capacity of more than 95% (inhibition of binding of BR55-2/murine IgG2a to the SKBR5 cell line).

Using the test procedures mentioned above six different hybridomas were finally selected and expanded (E4, C11, B3, B9, G6, G9). All six hybridomas produce murine IgG1 as detected by subtype ELISA using rabbit-anti-mouse IgG1/peroxidase (such as the reagent of Zymed).

All six hybridomas were cultured in roller flasks (37° C, 5% CO_2 in medium G; change of medium every 3 to 4 days) and the supernatants were collected for subsequent purification.

Each supernatant containing the respective anti-id BR55-2 Mab was purified using immunoaffinity chromatography. In general, affinity chromatography is based on the interaction between

an immobilized ligand and the substance of interest. In the case of anti-idiotypic BR55-2 Mabs, the highly specific ligand for the affinity column is Mab BR55-2/murine IgG2a which binds the anti-idiotypic Mabs of choice (for experimental details see example 5).

The degree of purity of the isolated anti-id BR55-2 Mabs (E4, C11, B3, B9, G6, G9) was tested by analytical FPLC ion-exchange-chromatography, size-exclusion-chromatography, SDS-PAGE and isoelectric focussing. Purity of all six anti-id BR55-2 Mabs was >95% (for experimental details see example 6; SDS-PAGE and isoelectric focussing are shown in figures 1 and 2).

The purified anti-id Mabs were quantitatively characterized by determination of their capacity to inhibit binding of BR55-2/murine IgG3 to the Lewis Y antigen positive SKBR5 cell line. All anti-id Mabs inhibit the binding of Ab1 to its antigen based on a 1:1 stoechiometry (for experimental details see example 7; representative results are shown in figure 3).

The final proof of the internal image properties of the anti-id BR55-2 Mabs described above and their use as surrogate for the Lewis Y carbohydrate antigen is based on their ability to generate an Ab3 response recognizing Lewis Y antigen positive cells in different species. According to the network theory of N. Jerne antibodies (Ab3) induced by immunization with internal image anti-id Mabs (Ab2) have a binding specificity similar to that of Ab1. Therefore the immune response evoked by immunization with anti-id BR55-2 Mabs should be specific for Lewis Y antigen positive HIV-infected cells. Consequently immunity to HIV may be induced in man by immunization with anti-id BR55-2 Mabs.

For the investigation of the properties of an Ab3 response rabbits as well as rhesus monkeys were immunized with anti-id BR55-2 #E4 using aluminium hydroxide as adjuvant. This mild adjuvant is widely used in different vaccines for human use. As a negative control the animals were also immunized with the same amount of unspecific mouse IgG1. After four immunizations during 5 weeks sera were collected at week 9 (for experimental details see example 8). Binding of serum Ig to the Lewis Y antigen positive SKBR5 breast cancer cell line and to the Lewis Y antigen negative WM9 melanoma cell line was determined (for experimental details see examples 9 and 10).

Anti-id BR55-2 #E4 elicits a high titered humoral immune response both in rabbits and rhesus monkeys. Serum Ig of animals immunized with anti-id BR55-2 #E4 selectively binds to the Lewis Y antigen positive tumor cell line but not to the Lewis Y antigen negative WM9 cell line. In contrast, by immunization with unspecific mouse IgG1 almost no tumor cell binding serum Ig is detected. These results are summarized in table 1. In figures 4 and 5 representative Ig-binding curves obtained with pre- and immunesera of rabbits and rhesus monkeys in a

cell-ELISA (SKBR5 cells) are shown. Binding of serum Ig of animals immunized with anti-id BR55-2 #E4 to SKBR5 cells can still be detected at a serum dilution of 1:10.000.

In order to proof the selective reactivity of serum Ig of rhesus monkeys immunized with anti-id BR55-2 #E4 to HIV-infected cells all monkey sera described above were tested in a commercially available test kit originally designed for detection of HIV seropositivity of human sera. This test is based on the ability of serum Ig to bind to the HIV-infected human T cell line PALL as detected by indirect immunofluorescence using anti-human IgG-FITC. Because of the great similarity of human and rhesus monkey Ig the original reagent of the test kit can be applied. Binding to non-infected PALL cells serves as control (for experimental details see example 11).

With all rhesus monkey sera the background immunofluorescence to both HIV-infected and non-infected PALL cells is slightly higher than the background immunofluorescence detected with normal human sera. However, only serum Ig of rhesus monkeys immunized with anti-id BR55-2 #E4 substantially binds to the HIV-infected PALL cells. The reactivity of serum Ig of rhesus monkeys immunized with unspecific mouse IgG1 to HIV-infected PALL cells is clearly less pronounced and close to the background immunofluorescence observed with all monkey sera (results are summarized in table 2).

In conclusion, immunization with anti-id BR55-2 Mabs leads to a high titered immune response which is specific for HIV-infected cells. Thereby the internal image properties of these anti-id BR55-2 Mabs and their use as surrogate for the Lewis Y carbohydrate antigen for therapeutic and prophylactic vaccination of man with the aim of induction of immunity against HIV is highlighted.

The following examples illustrate the invention. The abbreviations have the following meanings:

BSA:	bovine serum albumin
CDC:	complement dependent cytotoxicity
DMEM:	Dulbecco modified Eagle Medium
ELISA:	enzyme-linked immunosorbent assay
FCS:	fetal calf serum
Mab:	monolonal antibody
PBS:	phosphate-buffered saline
RPMI:	Rosewell Park Memorial Institute
SDS:	sodium dodecyl sulfate
KLH:	keyhole limpet hemocyanin
SPDP:	N-succinimidyl-3-(2-pyridyl-dithio-propionate)

PAGE: polyacrylamide gel electrophoresis

IEF: isoelectric focussing

PEG: polyethyleneglycol

The materials referred to in the examples are as follows:

Microtiterplates: Immunoplates II (Nunc)

Cell lines: SKBR5: human breast cancer cell line
PALL: human T cell line
SP2/0: mouse myeloma cell line

Medium B: RPMI 1640 + 2 g/l $NaHCO_3$
100 U/ml penicillin G
100 µg/ml streptomycin sulfate
4 mM glutamine
5 % FCS (heat-inactivated)

Medium C: DMEM
10 % NCTC-135 (synthetic medium, Gibco)
1 % MEM non essential amino acids (Gibco)
0.5 % sodium pyruvate
0.5 % oxalacetic acid (Sigma)
20 % FCS (heat-inactivated)
4 mM glutamine
100 U/ml penicillin G
100 µg/ml streptomycin sulfate

Medium D: Medium C + 1.36 mg/l hypoxanthine
0.39 mg/l thymidine

Medium E: Medium D + 0.4 mg/l aminopterin

Medium F: Medium C + mouse thymocytes (thymocytes of one Balb/c mouse resuspended in 25 ml medium C)

Medium G: DMEM
10 % FCS (heat-inactivated)
4 mM glutamine
100 U/ml penicillin G
100 µg/ml streptomycin sulfate

PEG: poly-ethylene-glycol (MW ≈ 3400)
1 g is dissolved in 1 ml DMEM

PBS deficient: 138.0 mM NaCl
1.5 mM KOH
2.7 mM KCl
6.5 mM Na_2HPO_4
pH 7.2

Coating buffer: 15 mM Na_2CO_3
35 mM $NaHCO_3$
3 mM NaN_3
pH 9.6

Staining buffer: 24.3 mM citric acid
51.4 mM Na_2HPO_4
pH 5.0

Washing buffer: 2% NaCl
0.2 % Triton X-100
in PBS deficient

Substrate solution: 40 mg o-phenylenediaminedihydrochloride
100 ml staining buffer
20 µl H_2O_2 30%

Binding buffer: 0.1 M Tris/HCl
0.2 M NaCl
pH 7.5

Elution buffer: 0.15 M glycine/HCl
0.2 M NaCl
pH 2.8

Coupling buffer: 0.1 M $NaHCO_3$
0.5 M NaCl
pH 8.0

In the following examples which illustrate the invention but in no way limit its scope references to temperature are in degrees celsius.

Example 1: Generation of anti-id BR55-2 #E4

1.1 Immunization of mice

Balb/c mice are immunized with each 100 µg of $F(ab')_2$-fragment of BR55-2/murine IgG3, coupled to KLH via SPDP as described (J. Carlsson et al., Biochem. J. 173, 723, 1978) by intraperitoneal injection in the following scheme:

day 0:100 µg of conjugate (1 mg/ml in PBS def.) + 100 µl of Freund's complete adjuvant

day 7 and 28:100 µg of conjugate (1 mg/ml in PBS def.) + 100 µl of Freund's incomplete adjuvant

On days 8, 9, 10 and 11 after primary immunization i.v. a total of 4 boost injections (each 100 µg of conjugate in 100 µl of PBS def.) are given.

On day 12 the spleens are taken out aseptically, suspended in PBS def. and washed thrice in PBS def.

1.2 Hybridization

These spleen cells are added to a suspension of SP2/0 cells in a ratio 1:1 and centrifuged at 900 g for 5 minutes. 1 ml of PEG-solution (37°) is added dropwise to the cell pellet within 1 minute and diluted with 1 ml of PBS def. (37°) within the next minute. 10 ml of medium C are added under gently rotation and the suspension is diluted to 50 ml with PBS def. The suspension is centrifuged at 800 g for 5 minutes, the pellet resuspended in medium D and the cells are transferred into the wells of a microtiterplate (Nunc 96) at a concentration of 2.5 x 10^5 cells/well. After overnight incubation at 37°/5% CO_2 100 µl/well of medium E are added. After 72 hours and then every four days the medium is replaced by medium D.

Example 2: Quantitative determination of mouse IgG in hybridoma supernatants

100 µl aliquots of rabbit-anti-mouse IgG (such as the reagent of Nordic; 1:1000 in coating buffer) are added to the wells of microtiter plates, and incubated at 37° for 60 minutes.

The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl aliquots of the hybridoma supernatants obtained after 2 weeks culture are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG/peroxidase such as the reagent of Dianova; 1:1000 in PBS/2% FCS) are added.

After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer.

100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Photometric extinction is measured at 492 nm (reference measurement 620 nm).

Example 3: Specific binding of hybridoma supernatant-IgG to BR55-2 F(ab')$_2$-fragment (ELISA)

Hybridomas producing sufficient mouse IgG (i.e. more as 10-fold optical density than the medium-blank) are subcloned to single cell culture in medium F and cultured in medium G for additional 2 weeks. The supernatants are tested as follows:

100 µl aliquots of F(ab')$_2$-fragment of BR55-2 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates, and incubated at 37° for 60 minutes.

The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl of hybridoma supernatants are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG-Fc/peroxidase such as the reagent of Dianova; 1:1000 in PBS/2 % FCS) are added.

After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer.

100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Photometric extinction is measured at 492 nm (reference measurement 620 nm).

Example 4: Inhibition of binding of BR55-2/murine IgG2a to SKBR5 human breast cancer cells by hybridoma supernatant-IgG (cell ELISA)

All hybridoma supernatants which are positive in the above described assay are tested as follows:

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml).

After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. Hybridoma supernatants adjusted to 1 µg/ml mouse IgG are preincubated with 10-fold excess of unspecific mouse IgG for 30 minutes at 37°. Then these samples are preincubated with 0.5 µg/ml of BR55-2/murine IgG2a for 30 minutes at 37°. 100 µl of this mixture are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05 % Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG2a/peroxidase such as the reagent of Zymed: 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes the colour development is stopped by addition of 50 µl aliquots of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 5: Immunoaffinity purification of anti-id BR55-2 Mabs

5.1 Preparation of BR55-2/murine IgG2a Sepharose

10 g of freeze dried activated CH-Sepharose 4B is suspended in 1mM HCl, transferred to a sinter glass filter and washed with 2 l of 1 mM HCl for 15 minutes. The ligand (120 mg of BR55-2/murine IgG2a) dissolved in 50 ml of coupling buffer is mixed with the washed gel in a stoppered vessel and rotated end over end for one hour at room temperature. The gel is washed with coupling buffer and incubated for one hour with 50 ml of 1M ethanolamine for blocking of any remaining active groups. The affinity sorbent is then washed with three cycles of alternating pH. Each cycle consists of a wash at pH 4 (0.1 M acetate, 0.5 M NaCl) followed by a wash at pH 8 (0.1 M Tris, 0.5 M NaCl).

5.2 Isolation of the anti-id BR55-2 Mabs

The chromatography is performed at 4°. The column (BIO REX MP column, diameter 1.5 cm) is filled with Mab BR55-2/murine IgG2a Sepharose (volume 35 ml). The gel is washed with binding-buffer and elution buffer. After equilibration with binding buffer conditioned medium containing anti-id BR55-2 is loaded onto the column at a flow rate of 15 ml/min. After elution of the breakthrough fraction, the bound anti-id BR55-2 is desorbed with elution buffer and neutralized immediately after desorption with 1 M Tris/HCl buffer, pH 7.5.

5.3 Concentration of the anti-id BR55-2 Mabs

Concentration of the eluted antibody solution (0.12 mg/ml) is performed in a stirred Amicon ultrafiltration cell using a PM 10 Diaflo membrane. The solute rejection for IgG is more than 98%, the final concentration of IgG amounts to 3.7 mg/ml.

Example 6: Characterization of purified anti-id BR55-2 Mabs

6.1 Ion-exchange-chromatography on Mono-Q

Column: Mono-Q HR5/5 (Pharmacia)
Buffer A: 20 mM tri-ethanolamine, pH 7.7
Buffer B: 20 mM tri-ethanolamine, 1 M NaCl, pH 7.7
Flow rate: 1 ml/min
Detection: UV 280 nm
Gradient: linear 2%/min
Results: > 95% purity found for all anti-id BR55-2 Mabs

6.2 High performance size-exclusion-chromatography

Column: Zorbax GF250, 9.4 x 250 mm
Buffer : Sodium phosphate 0.1 M, 0.2 M NaCl, pH 7.0
Flow rate: 1 ml/min
Detection: UV 280 nm
Results: > 95% purity found for all anti-id BR55-2 Mabs

6.3 SDS-PAGE

Experiments are performed both under reducing and non-reducing conditions according to the method of Laemmli using 10 % acrylamide gels (results are shown in Figure 1).

6.4 Isoelectric focussing

Analysis is performed with the Phast-system (Pharmacia) using a pH-gradient 3-9 (Phast gel IEF 3-9) and silver staining for detection of the protein bands (results are shown in figure 2).

Example 7: Binding of BR55-2/murine IgG3 to SKBR5 cell line (cell-ELISA) - Inhibition by anti-id BR55-2 #E4

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1 % in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05 % Tween 20. Anti-id BR55-2 #E4 is diluted in PBS def. containing 2% FCS (10 to 0.5 µg/ml). To each of these dilutions 1 µg/ml of BR55-2/murine IgG3 is added. 100 µl of

this mixture are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05 % Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (rabbit anti-mouse IgG3/peroxidase such as the reagent of Zymed; 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 8: Immunization of rabbits and rhesus monkeys with anti-id BR55-2 #E4

8.1 Immunization of rabbits with anti-id BR55-2 #E4

Three female chinchilla rabbits are immunized by intradermal application of 300 µg of anti-id BR55-2 #E4 adsorbed on aluminium hydroxide (1 mg of antibody plus 3.3 mg of $Al(OH)_3$/ml PBS def.) on days 1, 8, 15 and 36. Three rabbits are immunized with the same amount of unspecific mouse IgG1 as negative control under the same conditions. Sera are collected before immunization and at week 9 after first immunization.

8.2 Immunization of rhesus monkeys with anti-id BR55-2 #E4

Three rhesus monkeys are immunized by subcutaneous (s.c.) application of 0.1 mg of anti-id BR55-2 #E4/kg adsorbed on aluminium hydroxide (1 mg of antibody plus 3.3 mg of $Al(OH)_3$/ml PBS def.) on days 1, 8, 15 and 36. Two rhesus monkeys are immunized with the same amount of unspecific mouse IgG1 as negative control under the same conditions. Sera are collected before immunization and at week 9 after first immunization.

Example 9: Binding of rabbit serum Ig to SKBR5 cell line (cell-ELISA)

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. 100 µl aliquots of rabbit sera in appropriate predilutions are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05% Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-rabbit-Ig/peroxidase such as the reagent of Dianova; 1:1000 in

PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution is added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 10: Binding of rhesus monkey serum Ig to SKBR5 cell line (cell-ELISA)

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B (4×10^6 cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. 100 µl aliquots of rhesus monkey sera in appropriate predilutions are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05% Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human-Ig/peroxidase such as the reagent of Chemicon & Co., 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

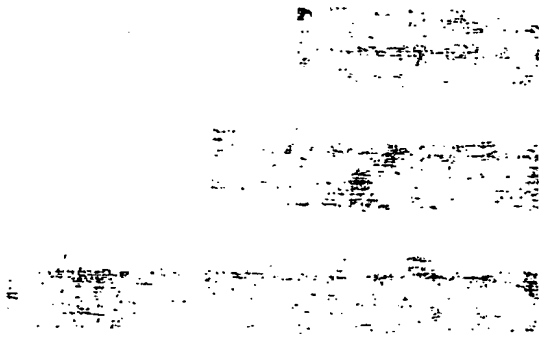
Example 11: Binding of rhesus monkey sera to HIV-infected/non-infected PALL-cell line (Indirect Immunofluorescence, IFA-Anti-HIV1-Kit Waldheim)

In a commercially available immunofluorescence assay kit (Waldheim & Co; purchased for determination of HIV seropositivity) HIV-infected and non-infected PALL cells (human T cell line) are fixed on slides. The experimental procedure is based on the guidelines of the supplier. After incubation with 1% BSA in PBS def. for 30 minutes at 37° the slides are incubated with rhesus monkey sera (concentrated and 1:10 diluted in PBS def.) for 1 hour at 37°. HIV positive human serum (delivered as part of the test kit) serves as positive control, normal human serum as negative control. Unbound determinants of the sera are washed out thrice with PBS def. and a 1:20 dilution of the anti-human-IgG-FITC reagent (part of the test kit) in PBS def. containing 2% FCS is added. After incubation for 30 minutes at 37°, washing thrice with PBS def.

and embedding of the slides the immunofluorescence is observed and scored in a fluorescence microscope.

In view of the above experimental results monoclonal anti-idiotypic BR55-2 Mabs are thus indicated for use in prophylactic and/or therapeutic immunization against HIV infections, especially against AIDS.

For the above-mentioned use the dosage will, of course, vary depending upon e.g. the compound employed, the subject patient's age, the stage of disease, the mode of administration or the treatment desired, and can be determined by the specialist in each individual situation. It will also vary when the antibodies are used in combination with chemotherapeutic agents like reverse transcriptase inhibitors or immunomodulating compounds. Administration is e.g. parenteral by subcutaneous injection together with a suited adjuvant like aluminium hydroxide. The dosage administrered is e.g. of from about 0.05 mg to about 10 mg of anti-id Mab as defined above. A typical administration schedule e.g. consists of 1 to 4 basic immunizations during 2 to 6 weeks and repeated boost immunizations e.g. every second month or on an indiviual basis after substantial decrease of Ab3 titers.



Claims:

1. Monoclonal murine internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1).

2. Process for the production of anti-idiotypic antibodies according to claim 1 which comprises immunizing mice with BR55-2/murine IgG3-F(ab')$_2$-KLH-conjugate, fusing the murine spleen cells with the murine myeloma cell line SP 2/0, selecting the cultured hybridoma cells which produce IgG with an inhibition capacity of more than 95% (inhibition of binding of BR55-2 murine IgG2a to the SKBR5 cell line), purifying and isolating the anti-idiotypic antibody.

3. Use of anti-idiotypic antibodies according to claim 1 for prophylactic and/or therapeutic immunization against HIV-infections.

4. A method of immunization against HIV-infections which comprises administering to a subject in need of such treatment a therapeutically effective amount of an anti-idiotypic antibody according to claim 1.

5. A pharmaceutical composition which comprises as active agent an anti-idiotypic antibody according to claim 1 together with a pharmaceutically acceptable adjuvant, carrier or diluent for use in the prophylactic and/or therapeutic immunization against HIV-infections.

Abstract:

Monoclonal murine internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1), process for their production and their use for immunization against HIV-infections.

SR/hz

Table 1

Immunization of rabbits and monkeys with anti-id BR55-2 #E4 or unspecific mouse IgG1

Relative titer increase at week 9 compared to 1:4 diluted preserum (Cell ELISA)

	Rabbit		Monkey	
	anti-id BR55-2	mouse IgG1	anti-id BR55-2	mouse IgG1
SKBR5 (Y^+)	1000	16	1000	8
WM9 (Y^-)	3	n.t.	25	n.t.

Table 2

Binding of rhesus monkey's sera to HIV-infected/non-infected PALL-cell line

(indirect immunofluorescence, IFA-Anti-HIV1-Kit Waldheim)

	Immunization of rhesus monkeys with				Controls (Kit)			
	anti-id BR55-2 #E4		unspecific mouse IgG		pos. control (HIV-pos. human serum)		neg. control (normal human serum)	
	+HIV-cells	-HIV-cells	+HIV-cells	-HIV-cells	+HIV-cells	-HIV-cells	+HIV-cells	-HIV-cells
0-sera								
conc.	1	1	1	1	5	0	0	0
1:10	1	1	1	1	-	-	-	-
sera 9 weeks after first immunization								
conc.	3	1	1-2	1				
1:10	3	1	1-2	1				

0-5 = intensity of fluorescence (anti-human IgG-FITC)

0 = no fluorescence
5 = maximum fluorescence

Figure 1
SDS - PAGE

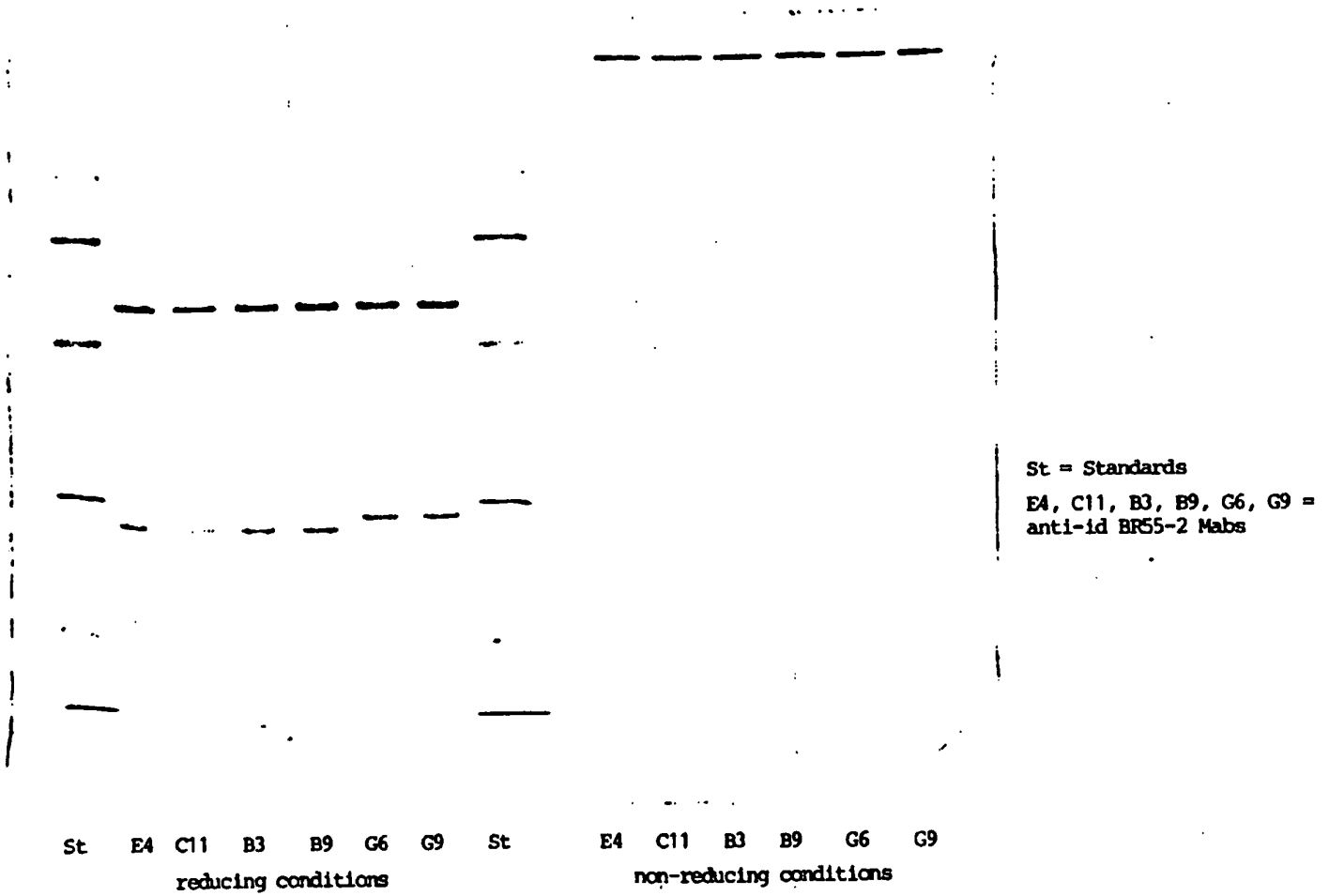
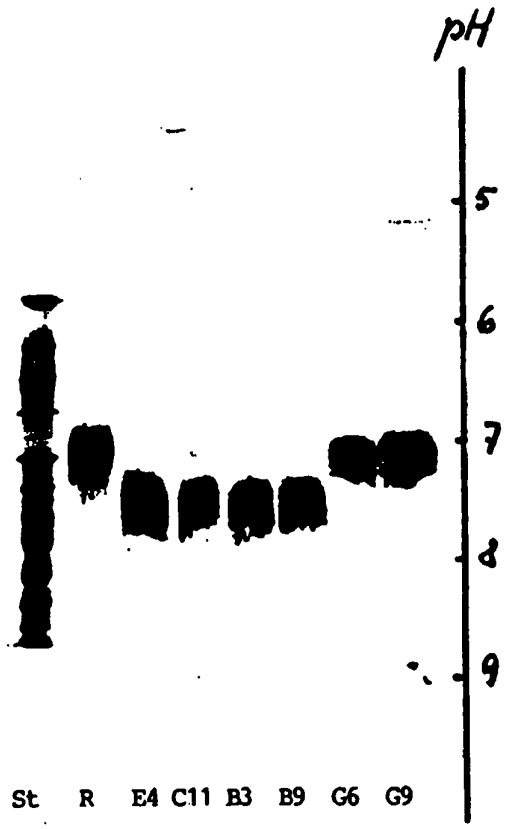
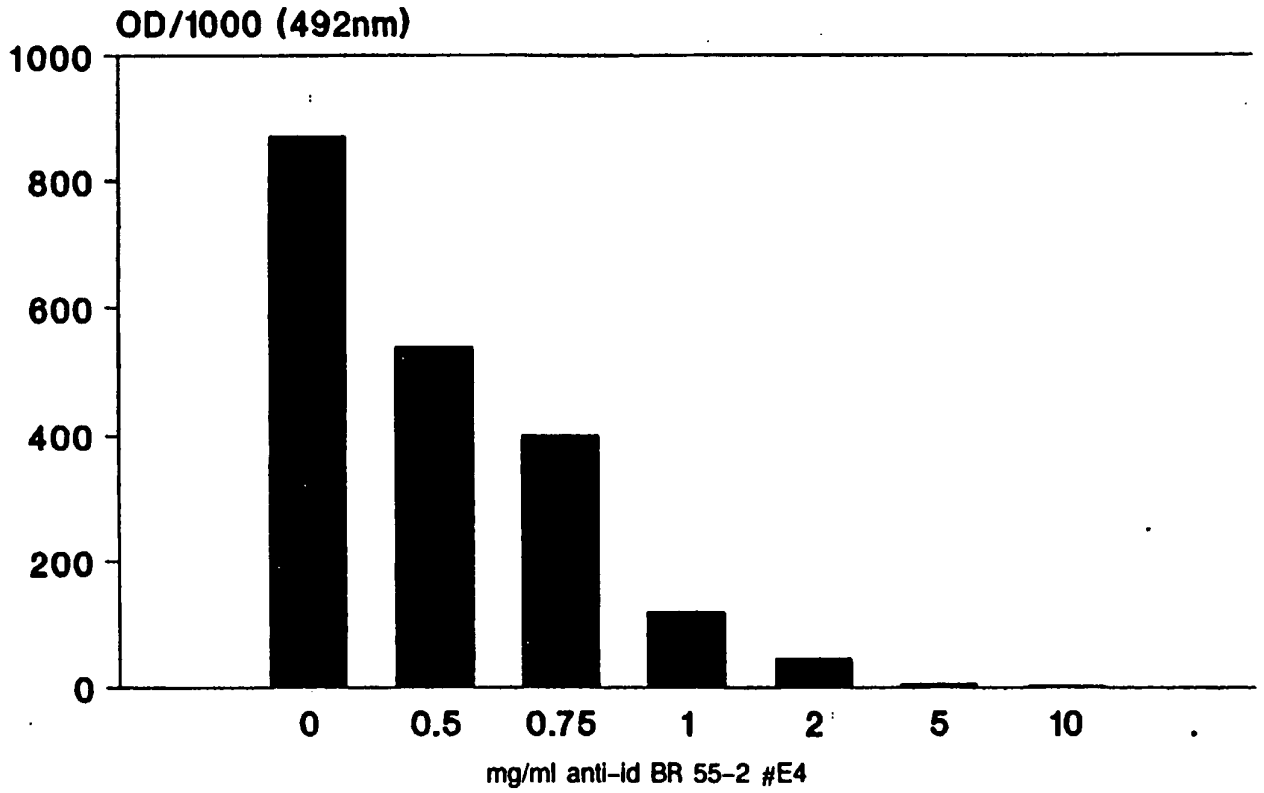


Figure 2
Isoelectric Focussing



St = Standard
R = Reference Mab
E4, C11, B3, B9, G6 and G9 = anti-id BR55-2 Mabs

Figure 3
Binding of BR55-2/murine IgG3 to SKBR5 cell line
Inhibition by anti-id BR55-2 #E4



BR 55-2/murine IgG3: 1 µg/ml

Figure 4
Binding of rabbit Ig to SKBR5 before and after immunization with anti-Id BR55-2 #E4 (Cell-ELISA)

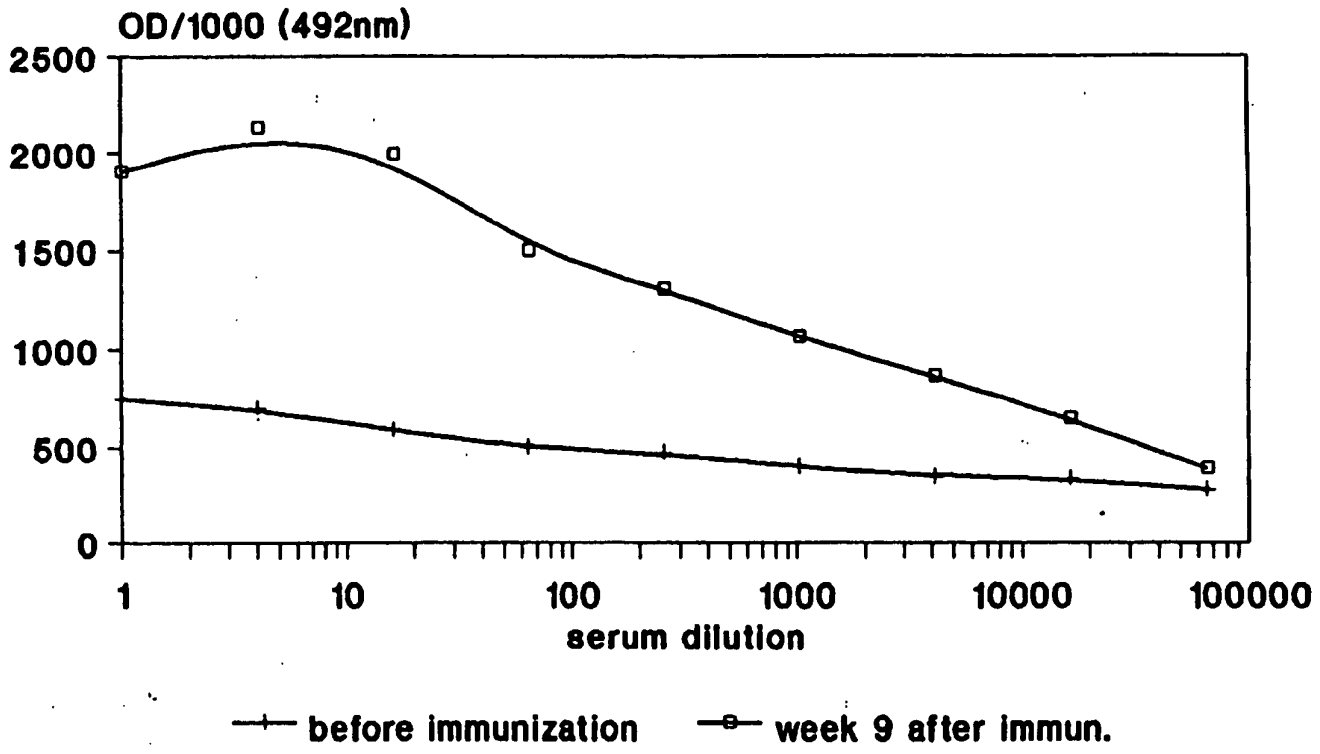
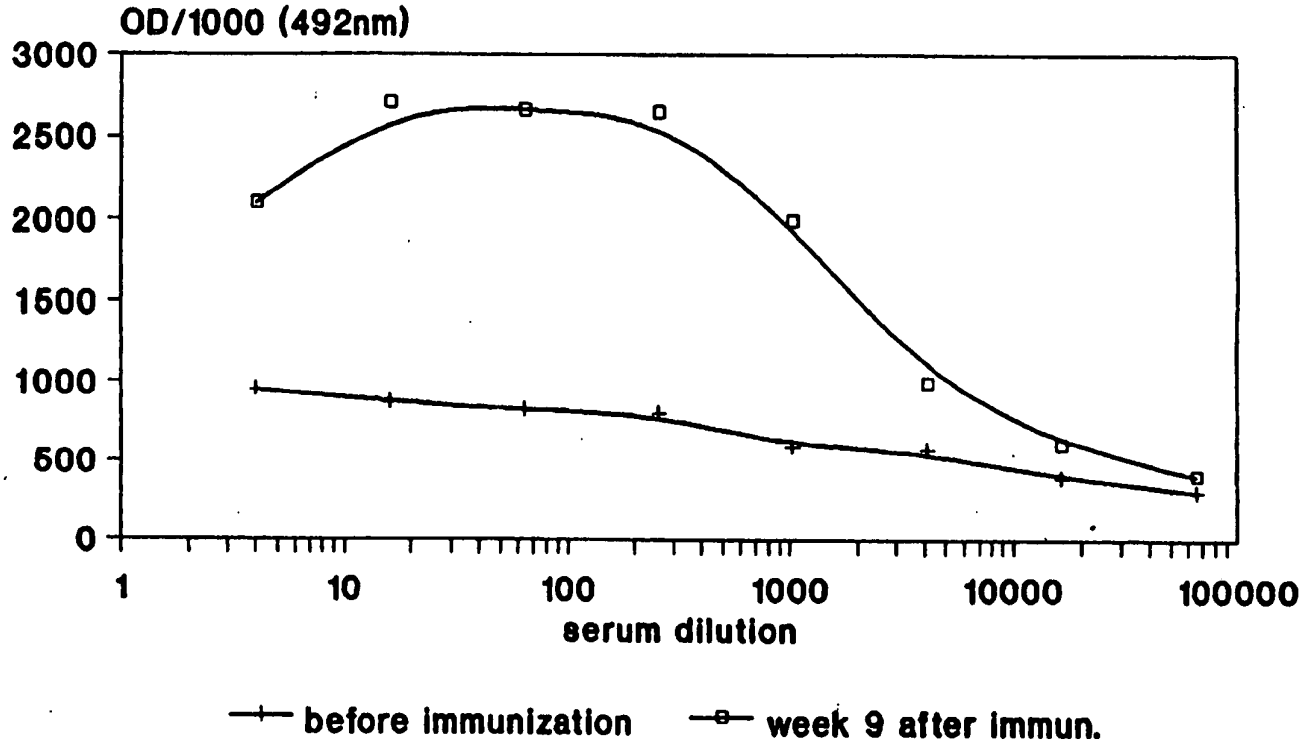


Figure 5
Binding of rhesus monkey Ig to SKBR5 before and after immunization with anti-Id BR55-2 #E4 (Cell-ELISA)



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